Enzyme Accessibility and Solid Supports: Which Molecular Weight Enzymes Can Be Used on Solid Supports? An Investigation Using Confocal Raman Microscopy

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Abstract: The accessibility of various solid supports (TentaGel, PEGA 1900, and beaded controlled pore glasses (CPGs)) to a range of enzymes was investigated. The different beaded materials were loaded with the peptide 4-cyanobenzamide-Gly-Pro-Leu-Gly-Leu-Phe-Ala-Arg-OH and incubated with the enzymes MMP-12 (22 kDa), thermolysin (35 kDa), MMP-13 (42.5 kDa), clostridium collagenase (68 kDa), and NEP (90 kDa). The absence/presence of the cyano stretching frequency was measured by means of confocal Raman microscopy. It was found that none of the investigated enzymes could enter the polymer matrices of TentaGel. PEGA 1900 was compatible only with the two smallest enzymes, while beaded CPG was successful even with NEP (90 kDa), proving its superiority over other materials in terms of bio-compatibility.

Keywords: combinatorial chemistry · enzymes · Raman spectroscopy · solid supports

Introduction

Solid-phase synthesis[1] has become very popular in recent years due to the growing importance of combinatorial chemistry,[2] with target compounds being prepared on the solid-phase either using parallel or split-and-mix based solid-phase methods,[3] or in solution with the appropriate use of reagent or scavenger-based resins.[4] Either way, the polymer matrix is a key requirement, yet many questions remain unanswered regarding the nature of the polymer materials being used. One problem of some practical interest is the applicability of enzymatic reactions on solid supports. In classical organic synthesis enzymes have been used for many years for a wide variety of chemical transformations, yet their application to solid-phase synthesis has been limited.[5] The most applicable supports for enzyme based-chemistry are the PEGA (cross-linked 2-acrylamidoprop-1-yl[2-aminoprop-1-yl]polyethylene glycol) based resins that have been championed by Meldal and co-workers.[6] However, in general, there has been much mystification in the solid-phase literature with respect to the applicability of enzymatically cleavable linkers on a number of other resin-based supports[8, 9] and bead-based enzyme screening, with no clear understanding of the compatibility of enzymes with synthesis based resin beads emerging. Table 1 gives a brief overview of the literature on the use of some enzymes that have been used in conjunction with synthetic based solid supports.

Work in our laboratory,[7a,b] and others,[7c] has highlighted the problem of carrying out fluorescent analysis on resin beads due to the inability (as a consequence of the Beer–Lamberts law) to illuminate more than a few microns into the bead when the bead is loaded with a dye or fluorophore.[7] As a consequence of this effect we therefore developed confocal Raman spectroscopy as a analysis method that is not sensitive to the concentration effects within the support, yet allows reaction sites on beads to be to probed with high levels of spatial, temporal and quantitative accuracy. This method therefore provides a powerful tool to allow the issue of enzyme accessibility into beads to be addressed.[7]

A study into the enzyme compatibility of some common supports was therefore carried out, with the focus being on the use of different PEGA, TentaGel, and beaded controlled pore glass (CPG) supports. PEGA can be prepared with different lengths of PEG cross-linkers with the number indicating the

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Table 1. Literature summary of some of the enzymes used on solid supports (TG = TentaGel, CPG = controlled pore glass, PEGA 1900 = polyethylene glycol(1900)-polyacrylamide copolymer.

Solid support	Enzyme	MW	Comments
TG	penicillin acylase	80 kDa	no cleavage of model compound[9]
PEGA 1900	penicillin acylase	80 kDa	13% cleavage of model compound[9]
CPG, pore size 50 nm	penicillin acylase	80 kDa	10% cleavage of model compound[9]
PEGA 1900	*E. coli* leader peptidase		FRET-based screening of a peptide library[10]
PEGA 4000	papain	23 kDa	FRET-based screening of a peptide library[6]
PEGA 4000 – 8000	MMP-9	67/83 kDa	FRET-based screening of a peptide library[11]
TG	lipase RB 001 – 05		enzyme-based safety catch linker 73% cleavage[12]
PEGA 1900	cruzipain	57 kDa	partial cleavage of resin bound peptide, mainly at the surface[13]
PEGA 800	cathepsin B & D	52 and 56 kDa	FRET-based screening of a peptide library[14]
TG	papain	23 kDa	little hydrolysis of model peptide[15]
ArgoGel	papain	23 kDa	
PEGA	papain	23 kDa	major cleavage of model peptide[15]
CPG	penicillin G acylase, phosphodiesterase	80 kDa	removal of a protecting group from oligonucleotides; nature of CPG not specified in the paper.[16]
TG	bovine chymotrypsin	22 kDa	1 – 2% segregation of the interior and the surface of the beads (shaving).[17]
	porcine elastase type I	22 kDa	2 – 13% note: repeated treatments were carried out with high levels of proteins
	porcine mucosa pepsin A	35 kDa	2 – 15% (1 mg mL^{-1})
PEGA 1900	galactosyltransferase	50 kDa	synthesis of glycopeptides[18]
TG	penicillin amidase	60 kDa	linker cleavage[19]

mass (and therefore length) of the PEG cross-linker. However only PEGA 900 and 1900 are commercially available, while the large size distribution of the beads and their tendency to stick together and to cling to the reaction vessel can make handling problematic. TentaGel, although swelling in water and easy to handle, has a polystyrene-based core and is based on the short cross-linking agent divinylbenzene at a level of 1 – 2% which should prevent access to most macromolecules for most practical applications. Few publications deal with CPG as a support for organic synthesis and enzymatic assay or cleavage. One of them described CPG from Fluka with a pore size of 50 nm in combination with a rather large enzyme (80 kDa), but only minor cleavage of a model compound from this support was detected and no other details of the support were given.[9] Other papers have not specified either the pore size of the CPG or the molecular weight of the enzyme used was unknown.[16] The material used in this study was a well-defined beaded CPG material used in large-scale protein purification processes.

Results and Discussion

To find the solid support most suitable for our needs the following experiment was carried out. The solid supports TentaGel, PEGA 1900, and beaded controlled pore glass (CPG, 100 nm pores) were loaded with the peptide 4-CBA-Gly-Pro-Leu-Gly-Leu-Phe-Ala-Arg-OH (4-CBA = 4-cyanobenzoic acid) and incubated with five enzymes with a range of molecular weights. The beads were investigated by confocal Raman microscopy to reveal if the peptide sequence was cleaved by looking at the stretching frequency of the cyano group at 2230 cm^{-1}. Table 2 and Table 3 show the relevant details of the solid supports and the enzymes used in this study.

Table 3. The enzymes and concentrations used in this study. The peptide sequence chosen for cleavage was 4-CBA-Gly-Pro-Leu-Gly-Leu-Phe-Ala-Arg-OH. Assay 50 mM Hepes, pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 1 μM $Zn(OAc)_2$.

Enzyme	Molecular weight [kDa]	Concentration μM
MMP 12	22	0.8
thermolysin	35	1.4
MMP 13	42.5	0.2
clostridium collagenase	68	5.0
NEP	90	0.5

To ensure that the peptide used on the different supports was of the same quality for all the samples, this peptide was first prepared in bulk on a polystyrene (PS) resin endowed with a 2-chlorotrityl linker (Scheme 1). Synthesis followed a conventional DIC/HOBt coupling procedure, with the guadinine side chain of arginine protected as its 2,2,5,7,8-pentamethyldihydrobenzofuran-5-sulfonyl amide (Pbf) and the peptide was designed to have a range of cleavage sites for a number of different proteases. The 2-chlorotrityl linker allowed cleavage of the peptide from the resin under

Table 2. The solid supports used in this study.[21]

Solid support	Bead diameter [μm]	Beads [g^{-1}]	Loading/bead	Comments
TentaGel	90	1 000 000	400 pmol	0.23 mmol g^{-1}
PEGA 1900	150 – 200	N/A[a]	~ 400 pmol	0.3 mmol g^{-1}
CPG 155	150 – 200	200 000	500 pmol	Johns Manville, pore size 100 nm, loading 0.18 mmol g^{-1}

[a] Not applicable as beads not available dried.

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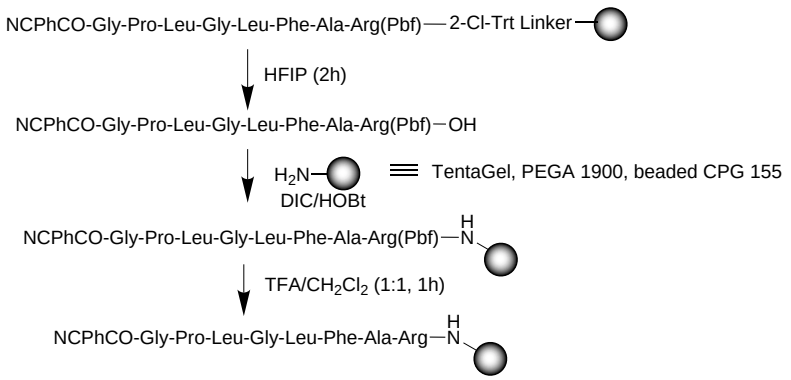

Scheme 1. Synthesis of the "general" protease substrate and attachment to the supports under investigation. DIC = *N*,*N*′-diisopropylcarbodiimide; HOBt = 1-hydroxybenzotriazole; TFA = trifluoroacetic acid.

extremely mild conditions using 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), and left the Pbf group of the arginine moiety intact (after each coupling step a small amount of the sample was cleaved from the resin and analysed by HPLC and mass spectrometry and was of good quality throughout). This allowed the peptide 4-CBA-Gly-Pro-Leu-Gly-Leu-Phe-Ala-Arg(Pbf)-OH to be coupled with DIC/HOBt onto the solid supports being investigated (until a negative ninhydrin test resulted), following which the Pbf group was cleaved with TFA/CH_2Cl_2 (1/1 for 1 h). Since the peptide was coupled to the solid supports without a linker, it was not possible to cleave for HPLC/MS analysis to check the successful removal of the protecting group. However analysis of the Raman spectrum revealed (Figure 1) that the Pbf group showed a small but very distinct vibration at 510 cm^{-1}, which was assigned to the SO_2 bending vibration of the Pbf moiety[20] which was present in Fmoc-Arg(Pbf)-OH and in the peptide coupled to the solid support. This stretch vanished following treatment with TFA. The cyano group was not attacked under these reaction conditions as could be seen from the signal at 2230 cm^{-1}.

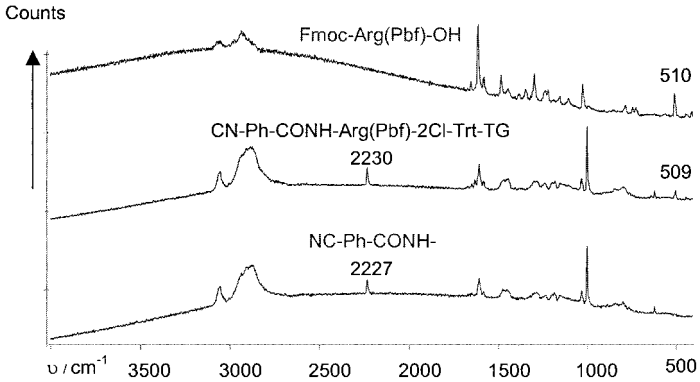

Figure 1. The SO_2 bending frequency of the Pbf group at 510 cm^{-1} and the nitrile tag at 2230 cm^{-1}.

Each of the solid supports bearing the peptide was initially investigated by Raman microscopy with the instrument set at the non-confocal mode, and spectra were collected in the range 2000–2600 cm^{-1} (Raman spectra were recorded by using a Renishaw 2000 system, set to the confocal mode, irradiation with a HeNe laser (633 nm), and a 1800 groove grating). The cyano stretching frequency could be clearly seen in each support. All resins were incubated overnight at room temperature with each of the enzymes, and the samples were washed with excess buffer and the Raman spectra recorded to give the data shown in Table 4 and Figure 2.

Table 4. Results of peptide cleavage. The statement "cleavage" and "no cleavage" are used to describe the absence or presence of the CN signal. (It is assumed that cleavage of up to 5% would remain unseen due to the S/N ratio).

	MMP 12	Thermolysin	MMP 13	Clostridium collagenase	NEP
TG	no cleavage	no cleavage	no cleavage	no cleavage	no cleavage
PEGA 1900	cleavage	cleavage	no cleavage	no cleavage	no cleavage
CPG 155	cleavage	cleavage	cleavage	cleavage	cleavage

It was immediately apparent that TentaGel, although swellable in water, was not accessible to any of the enzymes used. Even the small enzyme MMP 12 (22 kDa) had no detectable effect on the peptide. Thus any cleavage that takes place must be very limited (< 5%) and presumably "surface-based only". This result is in accordance with some of the literature on TentaGel,[9, 17] but in clear contradiction to other work in the area.[12, 19]

The comparison of our results for PEGA 1900 with the literature is difficult since many of the references do not state clearly the molecular weight of the enzymes used. The general trend in the literature seems to be that PEGA 1900 is partially accessible for enzymes with a molecular weight of up to 35–40 kDa. The cleavage of our peptide with MMP 12 and thermolysin on PEGA 1900 supports these findings. However larger enzymes are not permissible. With the beaded CPG used here complete cleavage of our peptide was observed

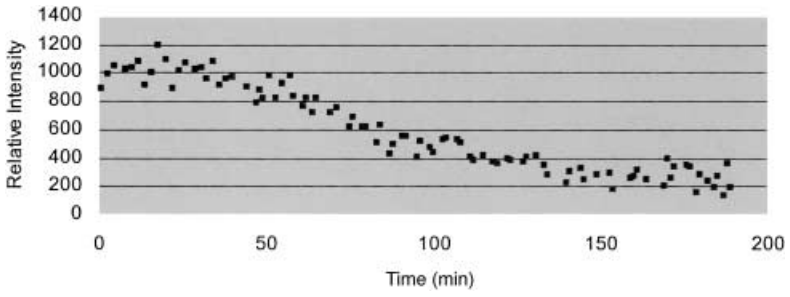

Figure 2. Single resin bead Raman spectroscopy analysis--peptide cleavage with MMP 0.8 μM, 50 mM Hepes, pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 1 μM $Zn(OAc)_2$.

even with NEP (90 kDa). This can be explained by the fact that in our studies we used glass beads with a pore size of 100 nm.

From our results it can be concluded that the support of choice for a broad number of enzymatic reactions on the solid phase was beaded controlled pore glass with a pore size of 100 nm. It offered complete accessibility to enzymes with a molecular weight of at least 90 kDa (in fact with pores of 100 nm enzyme accessibility is probably in the region of 1 MDa). Furthermore it is an easy to handle, non sticky material. It is inexpensive with a useable loading (0.1 – 0.05 mmol g^{-1}), while with 200 000 beads per gram it is ideal for split-and-mix synthesis. This support will now be one of the supports of choice for a range of resin-based enzymatic screenings and solid-phase combinatorial enzymatic transformations. The size accessibility and ease of handling means that beaded CPG is a very useful support for biological applications involving bead-based screening and combinatorial chemistry.

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